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Research Section

Disruption of sphingolipid metabolism in small intestines, liver and kidney of mice dosed subcutaneously with fumonisin B_1

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Abstract—Fumonisin B_1 is a fungal inhibitor of ceramide synthase, a key enzyme in the *de novo* biosynthesis of sphingolipids. The resulting increase in tissue free sphinganine (and sometimes sphingosine) is used as a biomarker for fumonisin exposure. This study determined whether a single subcutaneous injection of fumonisin B_1 could cause an increase in free sphingoid bases in the intestinal epithelial cells of mice over 24 hr. It was hypothesized that fumonisin administered subcutaneously would be excreted into the small intestine via biliary excretion, and this should be detectable by increased sphingoid bases in the intestine. A significant time-dependent increase in sphingoid bases occurred in the intestine and liver peaking at 4–8 hr and declining to control levels by 24 hr. In the kidney the increase in free sphinganine was persistent. The parallel time course of the change in sphinganine in the intestine and liver suggested fumonisin B_1 was rapidly excreted into the small intestine. Rapid cell turnover in the intestine could account for the reversal of the sphinganine increase. The rapid return to the control level in liver was unexpected since ceramide synthase inhibition in cultured cells is persistent suggesting that liver handles fumonisin B_1 or sphingoid bases quite differently than kidney. *Published by Elsevier Science Ltd.*

Keywords: fumonisin; *Fusarium moniliforme*; sphingolipids; liver; kidney; gastrointestinal.

INTRODUCTION

Fumonisins are mycotoxins produced by the fungus *Fusarium moniliforme* (synonym: *F. verticillioides*), and are commonly found in corn and corn products worldwide (Shephard *et al*., 1996). Consumption of corn-based feeds contaminated with high levels of fumonisins and pure fumonisin B_1 have been shown to cause farm animal diseases, such as equine leucoencephalomalacia (Kellerman *et al*., 1990; Thiel *et al*., 1991; Wilson *et al*., 1990) and porcine pulmonary edema (Harrison *et al*., 1990; Ross *et al*., 1991). In laboratory animals fumonisin B_1 is a liver and kidney carcinogen (Gelderblom *et al*., 1991; NTP, 1999), is hepatotoxic in all animals tested thus far, and nephrotoxic in rats, rabbits, sheep, pigs (for review see Dutton, 1996; Voss *et al*., 1996) and mice (Sharma *et al*., 1997). The occurrence of fumonisins in corn has been correlated with human esophageal cancer rates (Rheeder *et al*., 1992) and a relationship between fumonisin and liver cancer has been suggested (Ueno *et al*., 1997).

Fumonisins that contain a free amino group are inhibitors of ceramide synthase (sphinganine [sphingosine] *N*-acyltransferase) (Norred *et al*., 1997) a key enzyme in the *de novo* biosynthesis of sphingolipids (Fig. 1). Fumonisin B_1 inhibition of ceramide synthase causes a rapid increase in free sphinganine (Wang *et al*., 1991; Yoo *et al*., 1992), the immediate sphingoid base precursor in the *de novo* biosynthesis of dihydroceramide and ceramide. In animals, consumption of fumonisin B_1 disrupts sphingolipid metabolism as indicated by the accumulation of high

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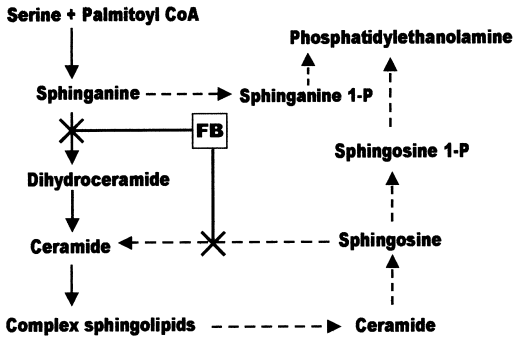

Fig. 1. Outline of the *de novo* sphingolipid biosynthetic pathway (left side, solid arrows) and the sphingolipid turnover and degradation pathway (right side, dotted lines) in an animal cell. The site of inhibition of fumonisin B_1, ceramide synthase (sphinganine (sphingosine) *N*-acyltransferase), is indicated with an "X". Complex sphingolipids include sphingomyelin and glycosphingolipids.

levels of free sphinganine in liver, kidney, serum and/or urine and the concomitant decrease in more complex sphingolipids (for review see Riley *et al.*, 1998). The increase in free sphinganine is useful as a functional biomarker for exposure of farm animals to toxic levels of fumonisins (Riley *et al.*, 1993; Wang *et al.*, 1992).

The fumonisin-induced elevation of free sphingoid bases correlates with increased cell death (apoptotic and oncotic) in liver and kidney *in vivo* (Tsunoda *et al.*, 1998) and, in cultured cells, inhibition of sphinganine accumulation reduces or prevents fumonisin-induced cell death (Riley *et al.*, 1999; Schmelz *et al.*, 1998; Tolleson *et al.*, 1999; Yoo *et al.*, 1996). Fumonisins can also inhibit apoptosis mediated by ceramide *de novo* in culture cells (Ueda *et al.*, 1998). Fumonisin B_1-induced apoptosis altered cell proliferation and altered signal transduction processes are believed to be linked to the fumonisin-induced changes in the biosynthesis of sphingoid bases, sphingoid base metabolites, ceramide, and more complex sphingolipids (for review see Merrill *et al.*, 1997).

In vivo, fumonisins are poorly absorbed and rapidly excreted. They are excreted in bile, thus, even though absorption is low, some enterohepatic recirculation occurs (Norred *et al.*, 1993; Prelusky *et al.*, 1996; Shephard *et al.*, 1994). The digestive epithelia of farm animals consuming low quality corn-base feeds can be exposed to high concentrations of fumonisins. Thus, the gastrointestinal tract may also be a target of fumonisins. As glycosphingolipids are attachment sites for microbial pathogens and their toxins (Karlsson, 1986), and fumonisin B_1 can reduce microbial toxin binding and transport in cultured cells (Sandvig *et al.*, 1996), inhibition of ceramide synthase in the digestive tract could alter the expression of glycosphingolipid attachment sites or microbial toxin transport and consequently the sensitivity of animals to infectious disease agents. In order to test the hypothesis that fumonisins inhibit de novo sphingolipid biosynthesis in the gastrointestinal tract, we needed a model system that would not be confounded by the presence of food in the digestive tract and avoided the problem associated with the rapid turnover of the epithelial cells in the gastrointestinal tract. It was hypothesized that because fumonisin B_1 is excreted in the bile following ip, iv or intragastric administration (Norred *et al.*, 1993; Prelusky *et al.*, 1996; Shephard *et al.*, 1994), fumonisin B_1 administered sc should be excreted in the small intestines via the bile and this should be detectable by elevation of free sphinganine in the epithelial cells of the small intestines. The purpose of this study was to determine the time course of changes in free sphingoid bases in liver, kidney and intestinal epithelial cells from mice treated with a single sc injection of fumonisin B_1.

MATERIALS AND METHODS

Animals and experimental

Male BALB/c mice, 20 g body weight and 6–7 wk of age, were obtained from Harlan Sprague–Dawley (Indianapolis, IN, USA) and acclimated for 1 wk in the animal facility. The animals were housed in groups of four each, provided with free access to fumonisin-free (< 1 ppm) commercial feed (Harlan Teklad 22/5 rodent diet, from Harlan-Teklad, Madison, WI, USA) and water. They were maintained in a controlled temperature (22°C) and humidity (45%) environment with a 12-hr light/dark cycle. Food and water intakes were recorded daily. Mice (four/time point) were injected once sc in the cervical region with 25 mg/kg fumonisin B_1 in sterile water (98% purity, Sigma Chemical Co., St Louis, MO, USA). Controls were injected with sterile water. Mice were euthanized with halothane at 0 (time-zero control), 2, 4, 8, 12 and 24 hr post dosing and the livers, kidneys and small intestines were removed. Liver and kidney were frozen on dry ice and then stored at −80°C for sphingolipid analyses.

The digestive epithelial cells of the small intestines were collected using a modification of the method of Weiser (1973). Briefly, a portion of the digestive tract (duodenum to caecum) was excised, and rinsed with 15 to 30 ml 0.154 M NaCl, containing 1 mM DTT to remove undigested materials. The washed intestines were ligated below the bile duct and filled with solution A (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 7.3). The open end was then ligated approximately 3 cm above the caecum, and the ligated intestinal segment incubated at 37°C. After 15 min, solution A was discarded, the intestinal segment filled with solution B (Hanks' buffered saline without calcium or magnesium, 1.5 mM EDTA, 0.5 mM DTT, pH 7.3), and incubated for 1 hr at 37°C. Thereafter, solution B containing the detached epithelial cells was collected

in a 15-ml conical tube. The intestinal segments were washed again with 10 ml of solution B to collect additional cells. The cells were pelleted by centrifugation at 900 ***g*** at 4°C, and after removal of the supernatant, stored at −80°C.

Sphingolipid analyses

Sphinganine and sphingosine in base-treated lipid extracts were determined by HPLC utilizing a modification (Riley *et al*., 1994) of the extraction methods originally described by Merrill *et al*. (1988). Sphingoid bases were quantitated based on the recovery of a C_{20}-sphinganine internal standard (generously provided by A.H. Merrill, Jr and D.C. Liotta, Emory University, Atlanta, GA, USA). A complete description of the HPLC apparatus and derivatization procedure is described in Riley *et al*. (1994). Sphingoid base concentrations were normalized either as a percent of the zero-time control values or the protein content using the bicinchoninic acid reagent (Pierce Inc. Rockford, IL, USA).

Inhibition of glycosphingolipid biosynthesis in rat intestines

The incorporation of 3-[^{3}H]D-erythro sphinganine (NEN Products, Boston, MA, USA) into glycosphingolipids and other complex sphingolipids isolated from Sprague–Dawley rat intestinal segments was determined to see whether fumonisin B_1 can directly inhibit ceramide synthase in the epithelial cells of the small intestines. Rats were used because they were readily available. Animals were housed and fed as described for mice in Material and Methods. Rats fasted for 16 hr were killed by CO_2 rather than halothane and the small intestines were removed and rinsed with Dulbecco's phosphate buffered saline (PBS) with 10 mM glucose, pH 6.5 (PBSG), and the segments were loaded with 1 ml PBSG that contained 1.4 μCi [^{3}H]sphinganine with (fumonisin-treated) or without (control) 50 μM fumonisin B_1 and incubated at 37°C and 90% relative humidity. After 2–4 hr, the intestines were cooled to 4°C, and the intestinal epithelial cells were removed by opening the intestines and scraping the mucosa with a spatula. The cells were collected in a tube, washed three times with cold PBS without glucose, pelleted at 900 ***g***, and freeze-dried. Glycosphingolipids were extracted using choloroform/methanol/water/pyridine (60:30:6:1, by vol.) by the method of Van Echten *et al*. (1990), followed by mild base hydrolysis to remove phospholipids and desalting using C-18 Sep-Paks (Waters, Milford, MA, USA). The isolated lipids were separated by silica gel thin-layer chromatography as described by Van Echten *et al*. (1990). Radioactivity on the developed thin layer chromatograms was detected using a Bioscan (Bioscan, Inc., Washington, DC, USA) radiochromatogram scanner and the areas corresponding to the radioactive peaks were compared to a series of commercially available unlabeled sphingolipid standards (Matreya, Inc., Pleasant Gap, PA, USA); ceramide, glucosylceramide, lactosylceramide, sphingomyelin, sphingosine and sphinganine. The non-radioactive standards were visualized by using iodine vapor. The areas corresponding to the sphingolipid standards and other radioactive peaks were scraped and counted using standard liquid scintillation counting methods.

Statistical analysis

Statistical analysis was done using Sigma Stat software (Jandel Scientific, San Rafael, CA, USA). One-way analysis of variance (ANOVA) was used followed by Dunnett's or Newman–Keuls test for *post hoc* multiple comparison. All data were expressed as mean ± SEM, and differences among means were considered significant if the probability was $\leqslant 0.05$.

RESULTS

All mice appeared healthy throughout the experiment. There was a significant ($P < 0.05$) time-dependent increase in free sphinganine concentration in the intestinal epithelial cells that peaked at 4–8 hr at approximately 350% of the time-zero control value and declined to the time-zero control level by 24 hr post dosing (Fig. 2A). Free sphingosine also increased; however, the difference was statistically significant only at 2 and 8 hr post dosing and, like the free sphinganine, sphingosine levels returned to control values by 24 hr (Fig. 2B). The change in the free sphinganine to free sphingosine ratio followed a similar pattern (Fig. 2B, inset).

In the liver, there was a statistically significant ($P \leqslant 0.05$) time-dependent increase in the free sphinganine level (Fig. 3A), which paralleled the time-dependent changes seen in the intestinal epithelial cells (Fig. 2A). However, the increase in free sphinganine peaked in liver earlier (2 hr) than in the intestinal epithelial cells (4 hr) and returned to near the control value by 12 hr (Fig. 3A). As in the intestinal epithelial cells, free sphingosine also increased significantly; however, the free sphinganine to free sphingosine ratio was not significantly different at any time point relative to the time zero control values (Fig. 3B, inset).

In the kidney, the increase in free sphinganine concentration was much greater than in either the intestinal epithelial cells or liver (Fig. 4A). For example, at 2 hr the mean free sphinganine concentration in liver and kidney was 87.2 and 437.1 pmol/mg protein, respectively. While the liver and intestinal epithelial cells reached maximal levels after 2 and 4 hr, respectively, the kidney reached its maximal increase at 12 hr. Even at 24 hr, when free sphinganine in both liver and intestinal epithelial cells were not significantly different than the time-zero control values, in the kidney the free sphinganine was 1156% of the time-zero control value (Fig. 4A). Likewise, the increase in free sphingosine (Fig. 4B)

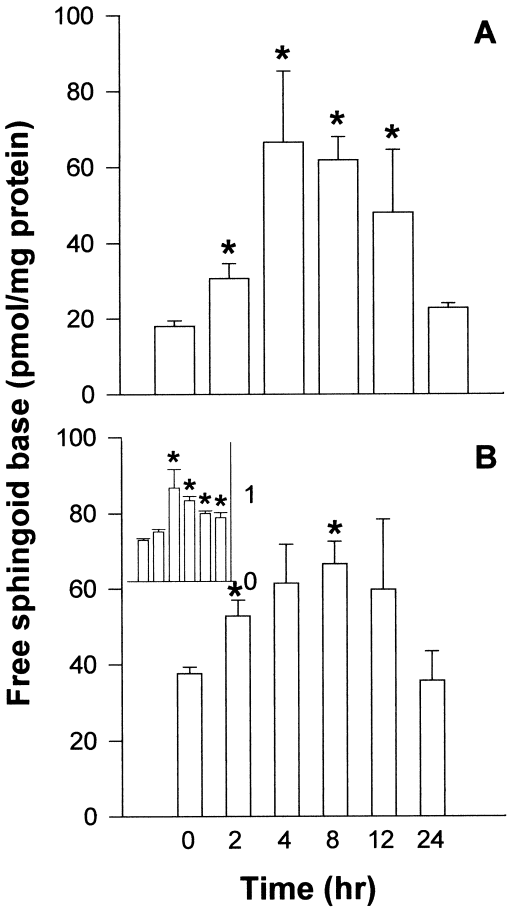

Fig. 2. The time course (0–24 hr) of the change in free sphinganine (2A), free sphingosine (2B), and the free sphinganine to free sphingosine ratio (Inset 2B) in small intestinal epithelial cells of mice dosed subcutaneously with fumonisin B_1 (25 mg/kg body weight). The time-zero mice were injected with sterile water. The values are expressed as the mean ± SEM (n = 4/time point). Asterisks indicate means that are significantly different from the time-zero control ($P \leqslant 0.05$).

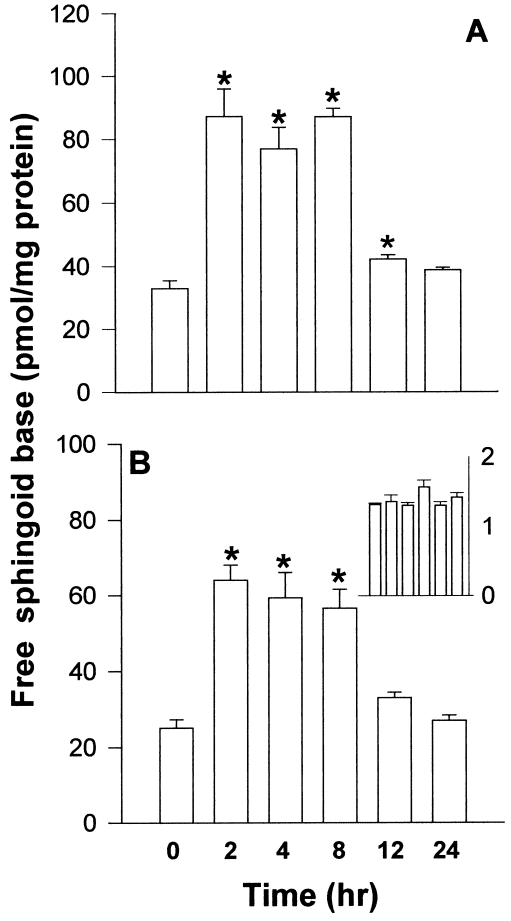

Fig. 3. The time course of the change in free sphinganine (3A), free sphingosine (3B), and the free sphinganine to free sphingosine ratio (Inset 3B) in liver. All other details as in Fig. 2.

and the free sphinganine to free sphingosine ratio (Fig. 4A, inset) in kidney were still significantly elevated at 24 hr relative to the time-zero control values (Fig. 4B and 4A inset).

Incorporation of 3-[^{3}H]D-erythro sphinganine into ceramide, lactosyl and glucosylceramide, and possibly sphingomyelin was decreased by at least 50% in the fumonisin-treated rat intestines relative to concurrent controls (no fumonisin added) (Fig. 5).

DISCUSSION

The inhibition of ceramide synthase (as evidenced by the increase in the upstream intermediate sphinganine) occurs very quickly after mice are dosed subcutaneously with fumonisin B_1. The increase in free sphinganine was most pronounced in the kidney, although, the increase occurred simultaneously in the liver and shortly thereafter in the intestinal epithelial cells. The results support the hypothesis that biliary excretion of fumonisin B_1 to the small intestines can inhibit ceramide synthase in the epithelial cells of the small intestines. In rats and pigs, biliary excretion and enterohepatic recirculation of fumonisin has been demonstrated (Norred *et al.*, 1993; Prelusky *et al.*, 1996; Shephard *et al.*, 1994). Alternatively, ceramide synthase inhibition could have occurred as a result of exposure via the submucosal vasculature or free sphinganine itself could be eliminated via the bile and accumulate in the epithelial cells in the absence of any fumonisin inhibition of ceramide synthase. Fumonisin B_1 inhibited 3-[^{3}H]D-erythro sphinganine incorporation into glycosphingolipids in isolated rat intestines (Fig. 5). Thus, in principle, fumonisins can directly inhibit sphingolipid biosynthesis in the small intestines; adding additional support to the notion that enterohepatic recirculation of fumonisins was the cause of the elevated free sphinganine in the mouse small intestine. None the less, the question still remains unanswered as to whether or not the intestinal epithelia is a target of

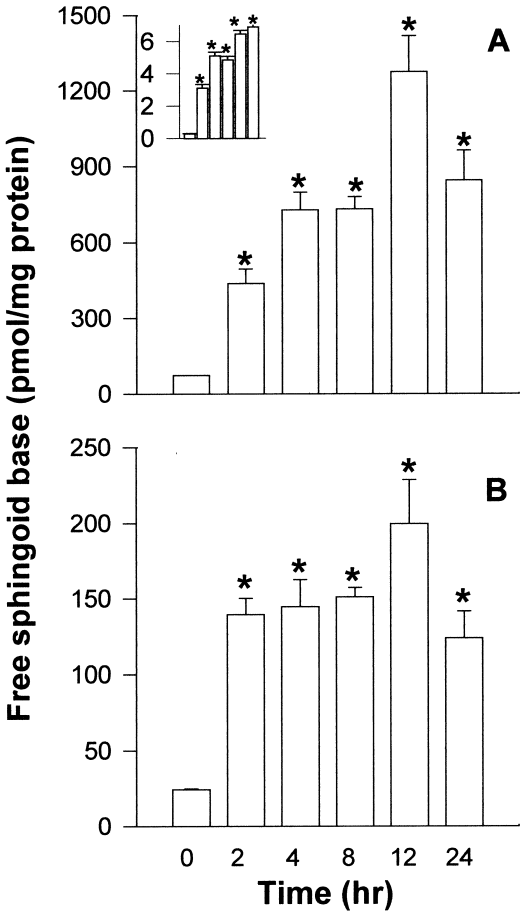

Fig. 4. The time course of the change in free sphinganine (4A), free sphingosine (4B), and the free sphinganine to free sphingosine ratio (Inset 4A) in kidney. All other details as in Fig. 2.

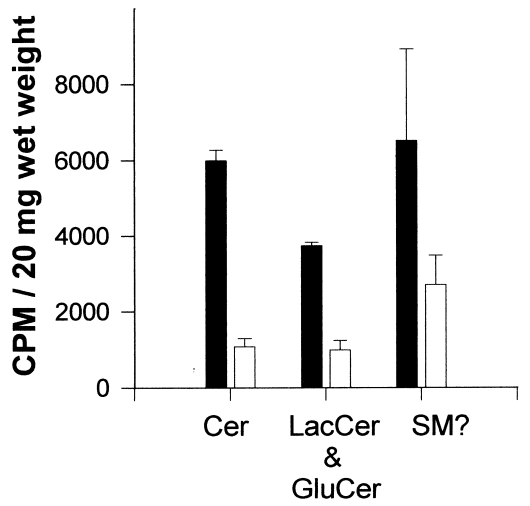

Fig. 5. The incorporation of 3-[^{3}H]D-erythro sphinganine (NEN products, Boston, MA) into ceramides (Cer), lactosyl- and glucosylceramide (LacCer & GluCer) and an unidentified lipid that migrated near the sphingomyelin standard (SM?) isolated from Sprague–Dawley rat intestinal segments. Values are the mean counts per min (cpm) from 20 mg fresh tissue ± SEM of duplicate intestinal segments, one from each rat. Black bars represent the control-, while white bars denote the fumonisin B_1-treated intestinal segments.

fumonisin B_1-induced ceramide synthase inhibition when exposed via the oral route.

Perhaps the most interesting finding of this study was that following subcutaneous administration of fumonisin B_1, elevated sphinganine levels in the liver and small intestines were rapidly reversible events while in the kidney the increase in free sphinganine was much more persistent. As hepatocytes are extremely sensitive to fumonisin-induced ceramide synthase inhibition (Norred *et al.*, 1996; Wang *et al.*, 1991), the transient increase in free sphinganine in liver suggests that subcutaneously administered fumonisin B_1 is rapidly eliminated by the liver and at least a portion is excreted into the small intestines via the bile. Alternatively, hepatocytes, and possibly the intestinal epithelial cells, are capable of either metabolizing or extruding sphingoid bases more efficiently than kidney. The rapid return to control levels in liver and small intestines was unexpected since ceramide synthase inhibition in cultured cells is quite persistent, whereas fumonisin is rapidly eliminated (Riley *et al.*, 1998; R.T. Riley, unpublished data). Part of the reason for the persistence of elevated sphinganine in the kidney may be because of a slower rate of elimination, relative to liver or small intestines, or the preferential accumulation in kidney when fumonisin is dosed subcutaneously. Interestingly, the kidney is a target for fumonisin B_1-induced apoptosis in mice dosed subcutaneously (Sharma *et al.*, 1997), an event that is closely correlated with the accumulation of free sphinganine (Tsunoda *et al.*, 1998), and apoptosis in kidney has also been observed in mice given fumonisin B_1 by gavage (Bondy *et al.*, 1997). However, in feeding studies, kidney toxicity was not clearly proven due to the low dose used in mice (NTP, 1999; Voss *et al.*, 1995). In the intestines the rapid rate of cell turnover could contribute to the transient nature of the elevation in free sphinganine in the intestines, a factor that will confound studies that attempt to detect evidence of fumonisin-induced ceramide synthase inhibition in animal feeding studies where animals are routinely fasted prior to sacrifice.

In conclusion, the results of this study demonstrate for the first time the rapid reversal of fumonisin-induced disruption of sphingolipid metabolism in the gastrointestinal tract, and strongly suggest that the cause of the altered levels of free sphingoid bases is the direct fumonisin inhibition of ceramide synthase in the intestinal epithelial cells. The data also show for the first time that the elevation in free sphingoid bases in liver is also rapidly reversed and, in contrast, that the accumulation of free sphingoid bases in kidney is much more persistent than in either liver or the intestine. As kidney is a target for fumonisin-induced apoptosis in mice dosed subcutaneously (Sharma *et al.*, 1997) but has not been demonstrated

in feeding studies (Voss *et al.*, 1995), the route of exposure and dosage chosen are important factors in determining the target organs in mice and possibly other species.

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